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Antisense oligodeoxynucleotides to the cystic fibrosis transmembrane conductance regulator inhibit cAMP-activated but not calcium-activated chloride currents

(patch clamping/channel regulation/epithelial cells)

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ABSTRACT **Phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) by cAMP-dependent protein kinase leads to chloride flux in epithelial cells. Is CFTR also required for the calcium-dependent activation of chloride channels? We used antisense oligodeoxynucleotides to CFTR to reduce the expression of CFTR in colonic and tracheal epithelial cells. The antisense oligomers were a pair of adjacent 18-mers complementary to nucleotides 1–18 and 19–36 of CFTR mRNA. Sense and misantisense oligomers served as controls. A 48-h antisense treatment reduced the expression of CFTR protein as assayed by immunoprecipitation and autoradiography to 26% of the level in sense-treated T84 cells. Whole-cell patch clamp revealed that a 48-h antisense treatment of T84 and 56FHTE-8o- fetal tracheal epithelial cells reduced the cAMP-activated chloride current to ≈10% of that in sense-treated cells. The half-life of functional CFTR is <24 h in these cells. In contrast, the calcium-activated chloride current was not affected by antisense treatment. Hence, the cAMP and calcium pathways are separate. CFTR is required for the cAMP pathway but not for the calcium pathway.**

Cystic fibrosis (CF), the most common lethal genetic disease in Caucasians, is associated with defective regulation of Cl^- secretion by epithelial cells (1). In CF cells, cAMP fails to induce Cl^- secretion (1–3). CF is caused by mutations in the gene for the CF transmembrane conductance regulator (CFTR; refs. 4 and 5). Expression of wild-type CFTR in CF cells restores cAMP-dependent Cl^- current regulation (6, 7). Moreover, epithelial and nonepithelial cells lacking endogenous CFTR acquire cAMP-regulated Cl^- current after transfection with the CFTR gene (2, 3). The selectivity and regulation of this current are altered by site-directed mutagenesis of the CFTR, demonstrating that phosphorylation of CFTR by cAMP-dependent protein kinase (PKA) followed by binding of ATP activates Cl^- movement (8–10). CFTR (11) or even its first nucleotide binding fold (12) act as Cl^--specific pores in lipid bilayers, establishing that CFTR is in fact a Cl^- channel.

Cl^- secretion in epithelial cells is also stimulated by the Ca^{2+}-triggered pathways, some of which are preserved in CF epithelial cells (13–16). Activation by Ca^{2+} is mediated by calmodulin and calcium/calmodulin-dependent protein kinase (CaMK) in T84 colonic epithelial cells and transformed normal and CF airway epithelial cells (16, 17). A variety of experimental findings, such as the preservation of Ca^{2+}- but not cAMP-dependent Cl^- flux in CF airway cells (13–16), imply that the Ca^{2+} pathway is independent of CFTR. This inference can be most directly tested by eliminating CFTR. We used antisense oligodeoxynucleotide treatment to lower the amount of CFTR in colonic (T84) and tracheal airway epithelial cells (56FHTE-8o-; ref. 16). Antisense treatment should then reduce the cAMP-activated Cl^- current without affecting the Ca^{2+}-activated Cl^- current. We monitored CFTR protein by immunoprecipitation followed by *in vitro* phosphorylation. Whole-cell patch clamp served to distinguish between cAMP-activated and Ca^{2+}-activated Cl^- currents.

MATERIALS AND METHODS

Oligonucleotides. Antisense oligodeoxynucleotide treatment has been used to block the expression of oncogenes and kinases (18). Antisense oligomers are typically directed to the initial nucleotides of the target mRNA. Cells are incubated with pairs of oligomers that are 15–22 nucleotides long (18). Longer oligomers do not readily enter cells, and shorter ones do not anneal to target mRNAs with sufficient specificity. The antisense oligodeoxynucleotides used in this study are a pair of adjoining 18-mers (5′-CAGAGGCGACCTCTG-CAT-3′ and 5′-GACAACGCTGGCCTTTTC-3′) that are complementary to nucleotides 1–18 and 19–36 of CFTR mRNA (nucleotide 1 begins with the AUG codon) (5). Two pairs of oligomers were used as controls. The pair of sense oligodeoxynucleotides (5′-ATGCAGAGGTCGCCTCTG-3′ and 5′-GAAAAGGCCAGCGTTGTC-3′) have a sequence corresponding to that of the mRNA (5). In addition, a pair of misantisense oligodeoxynucleotides (5′-CAGCGGCGAC-CGATGCAG-3′ and 5′-GACAACTCTGGACGTTTA-3′) were synthesized having the same sequence as the antisense pair except for the presence of four mismatches in each. Oligodeoxynucleotides were synthesized on an Applied Biosystems DNA synthesizer and then purified by HPLC and Sep-Pak C_{18} columns.

Cell Culture and Treatment. T84 cells were grown on glass coverslips or tissue-culture plastic flasks in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum (HyClone or GIBCO), 2 mM L-glutamate, penicillin (Biofluids, Rockville, MD; 100 units/ml), and streptomycin (Biofluids; 0.2 mg/ml). 56FHTE-8o- tracheal epithelial cells were grown on glass coverslips or tissue culture flasks in minimum essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamate, penicillin (100 units/ml), and streptomycin (0.2

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Abbreviations: CaMK, calcium/calmodulin-dependent protein kinase; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CPTcAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate; IP_3, inositol 1,4,5-trisphosphate; PKA, cAMP-dependent protein kinase.

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mg/ml). Oligodeoxynucleotide treatment for cells grown on coverslips for patch-clamp experiments was started at 30–40% confluency. Treatment for cells grown in flasks was started at 40–80% confluency, with the most consistent results obtained at lower confluencies. After the growth medium was removed, a pair of oligodeoxynucleotides (each at 20 μM) in medium without serum was added to the cells. After a 30-min incubation at 37°C with oligodeoxynucleotides, heat-inactivated serum (final concentration, 10%) was returned to the medium. The same procedure was used to replenish the oligodeoxynucleotides every 12 h.

Immunoprecipitation and *in Vitro* Phosphorylation. An equal number of T84 cells at 60–95% confluence (9.6 cm^2 per well) was solubilized with 2–5 ml of RIPA buffer [50 mM Tris·HCl/150 mM NaCl/0.1% Triton X-100/1% sodium deoxycholate/1% SDS/1 mM phenylmethylsulfonyl fluoride/*o*-phenanthroline (1 μg/ml)/aprotinin (10 μg/ml)/leupeptin (10 μg/ml)/pepstatin A (10 μg/ml), pH 7.5]. CFTR was immunoprecipitated from T84 lysates as described (9) by using the mouse anti-human monoclonal antibody EX13 (Genzyme). Immunoprecipitates were incubated with 66 ng of the catalytic subunit of PKA (Sigma) and 10 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq) in 50 μl of kinase buffer [50 mM Tris·HCl/10 mM $MgCl_2$/bovine serum albumin (0.1 mg/ml), pH 7.0] at 30°C for 60 min. The reaction was terminated by addition of 0.5 ml of RIPA buffer. Phosphorylated samples, adjusted for total protein content, were separated by SDS/PAGE. Protein was determined by the BCA assay (Pierce). Quantitative densitometry was carried out on a Molecular Dynamics Computing Densitometer.

Whole-Cell Patch Clamp. Cells were plated on glass coverslips and treated with oligodeoxynucleotide as above. Coverslips were placed in a 1-ml acrylic chamber on the stage of a Zeiss inverted microscope and washed six times with bath solution (170 mM Tris Cl/1 mM $MgCl_2$/2.5 mM $CaCl_2$/5 mM Hepes/10 mM glucose, pH 7.4, 320–330 milliosmoles/kg). Patch pipettes (19) had a tip resistance of 3–5 MΩ when filled with pipette solution (140 mM CsCl/2 mM $MgCl_2$/1 mM EGTA/2 mM MgATP/5 mM Hepes/10 mM glucose, pH 7.35 adjusted with CsOH, 295–298 milliosmoles/kg). Electrophysiologic experiments were all conducted at 30°C. Whole-cell voltage-clamp recordings were made as described (16). The membrane was voltage-clamped to a holding potential of −70 mV and stepped to levels between −100 and +100 mV in 50-mV increments. Voltage steps were 500 ms in duration with 750-ms intervals between steps. Variation in the plasma membrane area of different cells was taken into account by normalizing the current to cell membrane capacitance (pA/pF). Cells were monitored by whole-cell voltage clamp for at least 12 min after addition of 400 μM 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate (CPTcAMP), a membrane-soluble cAMP analog, or of 1 μM ionomycin, a calcium ionophore. A positive response was defined as a >2-fold increase in normalized current measured at +100 mV compared to baseline levels.

RESULTS

Effect of Oligomers on CFTR Protein in T84 Cells. Treatment of T84 cells with the antisense pair led to a reduction in the amount of measurable CFTR protein as assessed by autoradiography (Fig. 1). Glycosylated CFTR protein (apparent molecular mass, 183 kDa) decreased after 12, 24, 36, and 48 h of antisense oligodeoxynucleotide treatment to 92, 28, 41, and 26%, respectively, compared to the level in sense-treated cells. The corresponding values, compared to the level in untreated cells, are 48, 15, 21, and 14%, respectively. In contrast, after a 48-h incubation with sense or misantisense control oligomers, the level of CFTR was 52 and 78%, respectively, of that in untreated cells. The lower molecular mass band (150 kDa) is unglycosylated CFTR (20, 21); this band also

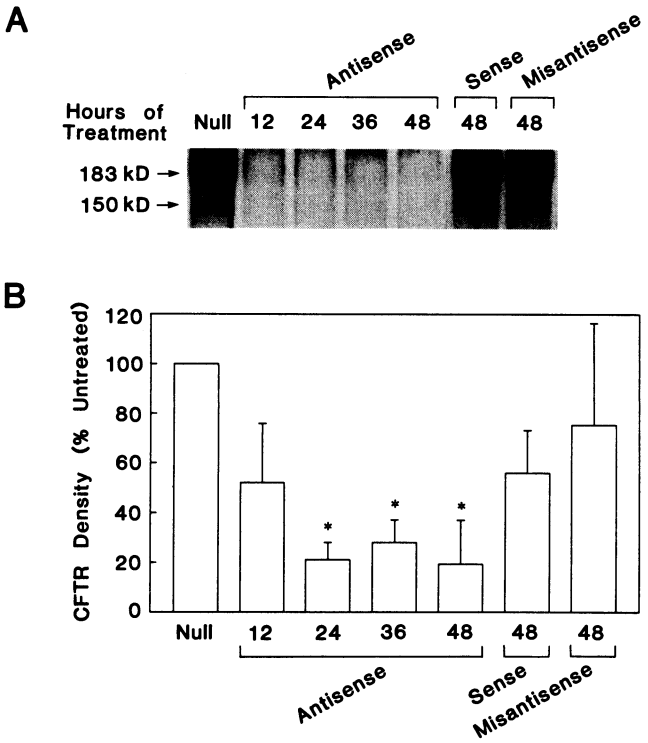

FIG. 1. Immunoprecipitation followed by *in vitro* labeling with PKA and [γ-^{32}P]ATP demonstrates that antisense oligodeoxynucleotides reduce the expression of CFTR protein in T84 cells. (*A*) CFTR protein was detected in untreated T84 cells and cells treated for 48 h with sense or misantisense oligodeoxynucleotides, but levels were reduced in cells treated for 12, 24, 36, and 48 h with antisense oligodeoxynucleotides. (*B*) Quantitative densitometry of extracts of cells receiving one of five oligomer treatments and subsequent immunoprecipitation and *in vitro* phosphorylation. Data are normalized to the density of untreated samples and expressed as mean ± SD. The number of quantitated bands for each treatment was 5, 5, 5, 4, 5, 3, and 4 for untreated, 12-h antisense, 24-h antisense, 36-h antisense, 48-h antisense, 48-h sense, and 48-h misantisense, respectively. Comparisons are by Student's *t* test. *, Statistically different from the sense-treated value ($P < 0.05$).

disappears in antisense-treated cells but is present in untreated and sense- and misantisense-treated cells. CFTR protein was not detected in autoradiograms of immunoprecipitates of lysates of 56FHTE-8o- cells (data not shown).

Effect of Oligomers on cAMP-Dependent Cl^- Current in T84 Cells. Untreated and sense- and misantisense-treated T84 cells had increased conductance in response to bath application of CPTcAMP (Fig. 2). Nearly all untreated and sense- and misantisense-treated cells responded to CPTcAMP (8 of 8, 7 of 8, and 7 of 7 cells, respectively). CPTcAMP increased the normalized current at +100 mV from 2.30 ± 1.21 (n = 18), 1.56 ± 0.675 (n = 16), and 3.39 ± 3.75 (n = 7) pA/pF to 104.0 ± 70.1 (n = 8), 68.5 ± 49.3 (n = 8), and 82.9 ± 31.3 (n = 7) pA/pF, in untreated and sense- and misantisense-treated T84 cells, respectively.

Measurement of reversal potentials established that outward current at depolarized potentials was carried predominantly by Cl^- ions. In all of the electrophysiological experiments, Na^+ was absent and K^+ currents were blocked by including 140 mM Cs^+ in the intracellular (pipette) buffer (16). The reversal potential in CPTcAMP-stimulated cells was −6.1 ± 8.1 mV (n = 8), in close agreement with the value of −5.4 mV calculated from the Nernst equation for Cl^-. The reversal potential shifted to −60.7 ± 19.1 mV (n = 3) when Cl^- was reduced to 15 mM in the internal solution, consistent with a calculated value of −64.5 mV. The voltage-clamp

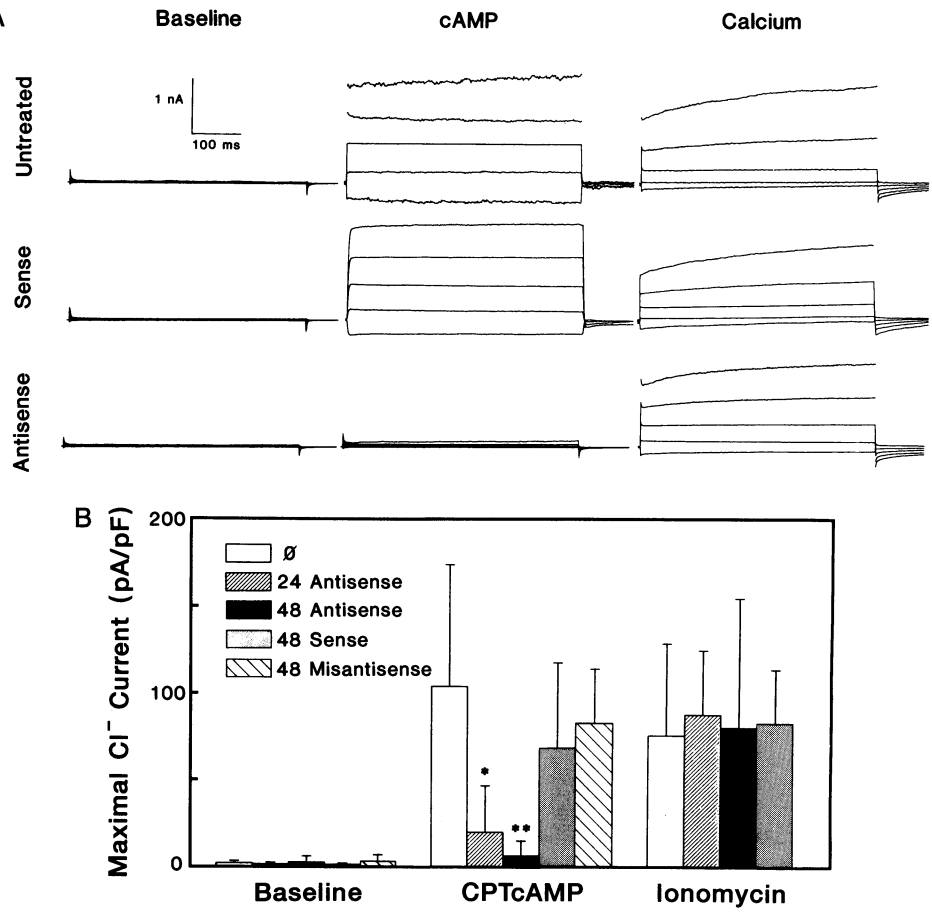

FIG. 2. Whole-cell patch-clamp experiments demonstrate that antisense oligodeoxynucleotide treatment reduces the level of cAMP-dependent Cl^- current but does not alter the amount of Ca^{2+}-dependent Cl^- current in T84 cells. (*A*) Representative whole-cell patch-clamp records. Rows: 1, untreated T84 cells; 2, sense oligodeoxynucleotide-treated (48 h) cells; 3, antisense oligodeoxynucleotide-treated (48 h) cells. Columns: 1, baseline Cl^- currents recorded 1–3 min after obtaining whole-cell configuration; 2, maximum Cl^- current recorded 1–12 min after addition of 400 μM CPTcAMP; 3, maximum Cl^- current recorded within 5 min after addition of 1 μM ionomycin. (*Inset*) Scale. (*B*) Maximal outward Cl^- current (mean ± SD). Bars: open, untreated T84 cells (n for baseline, CPTcAMP, and ionomycin = 18, 8, and 9, respectively); narrow hatched, cells treated for 24 h with antisense oligodeoxynucleotides (n = 16, 10, and 7, respectively); solid, cells treated for 48 h with antisense oligodeoxynucleotides (n = 15, 10, and 10); shaded, cells treated for 48 h with sense oligodeoxynucleotides (n = 16, 8, and 8); wide hatched, cells treated for 48 h with misantisense oligodeoxynucleotides (n = 7 and 7). *, Statistically different from untreated ($P < 0.01$), sense-treated ($P < 0.05$), and misantisense-treated ($P < 0.05$); **, statistically different from untreated ($P < 0.002$), sense-treated ($P < 0.005$), and misantisense-treated ($P < 0.002$). Comparisons are by Student's t test.

record displayed little time dependence and the steady-state whole-cell current–voltage relation was essentially linear, as observed for T84 (15) and airway epithelial cells (16).

Incubation with antisense oligomers diminished both the number of cAMP responders and the maximal amount of cAMP-dependent Cl^- current. Four of 10 cells treated for 24 h with antisense oligomers did not respond at all to CPT-cAMP. The mean maximal response of the 10 cells was only 29% of that of sense-treated cells. Eight of 10 cells treated for 48 h with the antisense pair showed no response, and the mean maximal response of the 10 cells was only 10%.

Effect of Oligomers on Ca^{2+}-Dependent Cl^- Current in T84 Cells. Whole-cell currents induced by ionomycin, a Ca^{2+} ionophore, are shown in Fig. 2. These voltage-clamp records display a slow time-dependent activation at strongly depolarizing potentials. Also, the steady-state current–voltage relation shows greater outward rectification than was observed for the cAMP-induced Cl^- current, as observed in T84 (15, 17) and airway epithelial cells (16). The reversal potential for whole-cell ionomycin-induced Cl^- conductance was -10.1 ± 6.1 mV (n = 9) in untreated cells. Reduction of the pipette Cl^- concentration to 15 mM resulted in a shift of the reversal potential to -49.0 ± 10.1 mV (n = 3), similar to that predicted by the Nernst equation for Cl^- as the permeant species.

Antisense oligomers had essentially no effect on the ionomycin-induced Cl^- current, in contrast to their effect on the CPTcAMP-induced Cl^- current (Fig. 2). All untreated and 24-h and 48-h antisense- and sense-treated cells responded to ionomycin (n = 9, 7, 10, and 8, respectively). The normalized current at +100 mV increased to 75.7 ± 53.3 (n = 9), 87.7 ± 37.3 (n = 7), 80.4 ± 74.6 (n = 10), and 83.2 ± 30.8 (n = 8) pA/pF for untreated and 24-h antisense-treated, 48-h antisense-treated, and 48-h sense-treated cells, respectively, after treatment with ionomycin.

Effect of Oligomers on Cl^- Currents in 56FHTE-8o- Cells. Similar results were obtained in 56FHTE-8o- cells (Fig. 3). CPTcAMP increased the normalized Cl^- current in sense-treated 56FHTE-8o- cells from 1.59 ± 0.85 to 40.0 ± 23.2 pA/pF in four of four cells, a level similar to that previously observed (16). Incubation with antisense oligomers reduced the amount of cAMP-dependent Cl^- current and the number of cAMP-responders. Three of five cells treated for 48 h with antisense oligomers did not respond to CPTcAMP, and the mean maximal response of the five cells was only 10% of that of sense-treated cells. In contrast, antisense oligomers had no effect on whole-cell Cl^- in response to the Ca^{2+} ionophore (Fig. 3). Ionomycin increased the normalized Cl^- current at +100 mV from 1.43 ± 0.84 and 2.42 ± 1.58 pA/pF to 70.7 ±

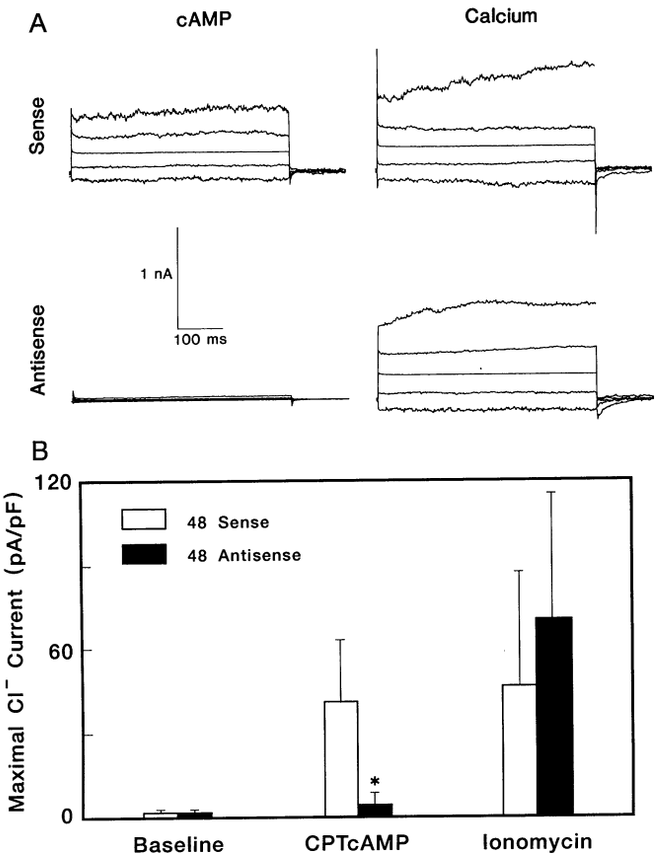

FIG. 3. Whole-cell patch-clamp measurements demonstrate that antisense oligodeoxynucleotide treatment reduces the level of cAMP-dependent Cl^- current but does not alter the amount of Ca^{2+}-dependent Cl^- current in 56FHTE-8o- cells. (*A*) Representative whole-cell patch-clamp records. Rows: 1, sense oligodeoxynucleotide-treated (48 h) cells; 2, antisense oligodeoxynucleotide-treated (48 h) cells. Columns: 1, maximum Cl^- current recorded 1–12 min after addition of CPTcAMP; 2, maximum Cl^- current recorded within 8 min after addition of 1 μM ionomycin. (*Inset*) Scale. (*B*) Maximal outward Cl^- current (mean ± SD). Bars: open, 56FHTE-8o- cells treated for 48 h with sense oligodeoxynucleotides (n for baseline, CPTcAMP, and ionomycin = 8, 4, and 4); solid, cells treated for 48 h with antisense oligodeoxynucleotides (n = 10, 5, and 5). *, Statistically different from sense-treated ($P < 0.05$) by Student's t test.

44.5 (n = 5) and 45.9 ± 41.9 (n = 4) pA/pF in 48-h antisense- and sense-treated 56FHTE-8o- cells, respectively.

DISCUSSION

Antisense oligodeoxynucleotides to CFTR markedly reduced the amount of CFTR protein in T84 cells. The observation that the amount of CFTR protein was lowered to 14–24% of the level in untreated cells after a 24- to 48-h incubation with antisense oligomers (Fig. 1) implies that the half-life of CFTR protein is somewhat shorter than 24 h in these cells. These data are consistent with those of Sorscher *et al.* (22) who reported that CFTR protein had a turnover time of <24 h, as shown by halide-specific fluorescence imaging of antisense-treated cultured sweat duct cells. A similar time course was observed by Cheng *et al.* (21) after transfection of COS cells with CFTR.

Our patch-clamp studies of colonic and airway epithelial cells demonstrate that antisense treatment reduces cAMP-activated Cl^- current. The current in T84 cells was lowered to 29 and 10% of the control value after 24- and 48-h incubations, respectively (Fig. 2*B*). These kinetics indicate that the half-life of functional CFTR is <24 h, in agreement with the estimates cited above. In T84 cells, the amount of CFTR protein and the magnitude of the cAMP-dependent Cl^- current are strongly correlated (r = 0.98; $P < 0.001$). However, the relationship between CFTR protein and cAMP-dependent Cl^- current in airway cells is uncertain. We and others (20, 21) have not detected CFTR protein in airway cells by immunoprecipitation and *in vitro* phosphorylation. Nevertheless, we observed a large inhibition of cAMP-activated current by antisense treatment.

In contrast to cAMP-stimulated Cl^- currents, Ca^{2+}-activated Cl^- current was unaffected by antisense treatment in both colonic and airway epithelial cells. The following lines of experimental evidence suggested that the cAMP and Ca^{2+} pathways activate separate Cl^- conductances in epithelial cells: (*i*) whole-cell current–voltage relations, (*ii*) time dependence of whole-cell currents, (*iii*) relative halide selectivity, (*iv*) differential inhibition by anionic blockers, (*v*) additivity of cAMP-induced and Ca^{2+}-induced currents, and (*vi*) preservation of Ca^{2+}-dependent but not cAMP-dependent Cl^- flux in CF airway epithelial cells (13–16, 23). Although these differences could have been attributed to separate transport proteins, differences in current–voltage relations, selectivity profiles, and gating properties could also result from different multimeric structures or covalent modification of the same protein (24, 25). The preservation of Ca^{2+}-activated Cl^- current in cells in which CFTR has been reduced by antisense treatment definitively demonstrates that the cAMP and Ca^{2+} pathways are also separate with respect to the involvement of CFTR; specifically, in both colonic and airway epithelial cells, CFTR is required for the cAMP pathway but not for the Ca^{2+} pathway. Antisense treatment may prove useful as a means of identifying other CFTR-independent Cl^- flux pathways.

The flow of information in these parallel pathways for the activation of Cl^- channels is shown in Fig. 4. The cAMP pathway is triggered by the binding of a hormone to receptor 1 (e.g., epinephrine to a β-adrenergic receptor). The stimulatory guanine nucleotide-binding (G) protein G_S then stimulates adenylate cyclase to form cAMP. Increased cAMP levels result in the activation of PKA, which in turn phosphorylates and increases the open probability of the CFTR Cl^- channel (9, 11). In CF, the amount of functional CFTR is apparently diminished (26, 27). The Ca^{2+} pathway is triggered by the binding of a hormone to receptor 2 (e.g., acetylcholine to a muscarinic receptor). A phospholipase C-specific G protein then activates phospholipase C, which in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 releases Ca^{2+} from stores in the endoplasmic reticulum. Ca^{2+}–calmodulin activates CaMK, which phosphorylates and, thereby, increases the open probability of the Ca^{2+}-dependent Cl^- channels (16, 17). The Ca^{2+} pathway for Cl^- channel regulation operates normally in CF airway epithelial cells (13–16).

The presence of a normal Ca^{2+} pathway in CF airway epithelial cells suggests a potential means of circumventing the CF defect. The challenge is to activate the Ca^{2+} pathway to generate Cl^- flux without triggering adverse side effects. Knowles *et al.* (28) have found that ATP and UTP acting through nucleotide P_2 receptors increase Cl^- secretion in the airway of CF patients and normal controls. Nucleotide P_2 receptors are known to activate phospholipase C and cause an accumulation of IP_3 and a consequent increase in intracellular Ca^{2+} (29). It seems likely that CaMK then mediates Cl^- channel opening (16, 17), as depicted in Fig. 4. The increase in intracellular Ca^{2+} caused by nucleotide triphosphates is short-lived (ref. 30; A. C. Chao and P.G., unpublished observations), but the activation of Cl^- current is long-lasting (28). A brief Ca^{2+} transient may activate CaMK for a much longer duration by inducing its autophosphorylation, which makes it active even at low Ca^{2+} (31). Alternatively, CaMK-activated Cl^- channels may account for only

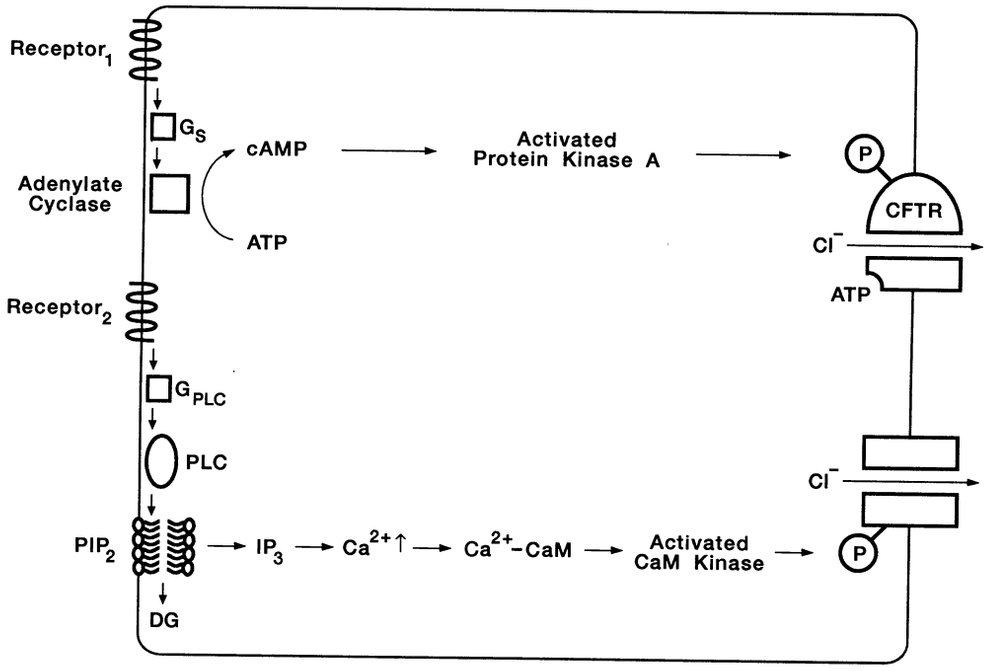

FIG. 4. Control of plasma membrane Cl^- channels by the parallel cAMP and Ca^{2+} pathways in an idealized airway epithelial cell. PLC, phospholipase C; G_S and G_{PLC}, stimulatory and PLC-specific guanine nucleotide binding proteins, respectively; PIP_2, phosphatidylinositol 4,5-bisphosphate; DG, diacylglycerol; CaM, calmodulin.

the initial part of the response to nucleotide triphosphates; a direct effect of nucleotide P_2 receptors on an apical Cl^- channel (32) may produce the sustained part of the response. A number of agents in addition to ATP and UTP increases epithelial intracellular Ca^{2+} and activates Cl^- current. Unfortunately, muscarinic acetylcholine receptor agonists and bradykinin (30, 33) cause undesirable side effects such as bronchoconstriction (34). Likewise, extracellular ATP is rapidly hydrolyzed to adenosine, which induces bronchoconstriction. UTP, on the other hand, does not yield bronchoconstrictive breakdown products and, hence, is a good candidate for further study.

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